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(54) **OLIGONUCLEOTIDES USED FOR DETECTING VIBRIO PARAHAEMOLYTICUS AND METHOD OF DETECTION THEREWITH**

(57) An oligonucleotide having a nucleic acid sequence derived from SEQ ID NO:1 and at least one site capable of amplifying a nucleic acid sequence characteristic of *Vibrio parahaemolyticus*; the above oligonucleotide having a nucleic acid sequence unavailable from SEQ ID NO:3; the above oligonucleotide incapable of amplifying nucleic acid sequences originating in *Vibrio alginolyticus* and *Vibrio harvei*; the above oligonucleotide represented by the sequence of CGG CGT GGG TGT TTC GGT AGT or TCC GCT TCG CGC TCA TCA ATA; and a method of detecting *Vibrio parahaemolyticus* by preparing a primer set comprising two of the above oligonucleotides, selectively amplifying therewith a DNA gyrase subunit B gene sequence contained in a specimen as a target, and determining whether or not there is a *gyrB* unit specific for *Vibrio parahaemolyticus* in the specimen. This method has made it possible to provide a primer which specifically reacts with a *gyrB* gene of *Vibrio parahaemolyticus* to thereby differentiate and identify the same among other vibrios and strains other than the genus *Vibrio*. The primer specific for *Vibrio parahaemolyticus* serves to detect 285-bp *gyrB* gene fragments specific for this vibrio by the PCR method without the necessity for DNA extraction or like operations from bacterial cells.

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## Description

## FIELD OF THE INVENTION

[0001] This invention relates to oligonucleotide primers for amplification of the target nucleotide sequence characteristic of *Vibrio parahaemolyticus* (abbreviated as "VP" somewhere hereinafter). This invention relates to the method for detecting *Vibrio parahaemolyticus* based on the polymerase chain reaction (PCR) using a primer specific for the DNA gyrase sub-unit B gene (Nucleotide sequence of DNA gyrase B subunit, abbreviated as "gyrB" hereinafter).

## PRIOR ART

[0002] *Vibrio parahaemolyticus* is known to cause food poisoning in many countries. It is found not only in the intestine but also in other organs and in the postoperative wound. *Vibrio parahaemolyticus* is a Gram negative, polymorphic, bacilliform, halophilic, facultative anaerobe, which ferments carbohydrate to generate gas. It forms green colonies on thiosulfate-citrate-bile-sucrose (TCBS) agar.

[0003] For detection of *Vibrio parahaemolyticus*, is used usually a method where the specimen is cultivated in an enrichment medium followed by isolation by the selective plate culture. The conventional method of detection requires one week, and therefore a more rapid method has been desired.

[0004] The fluorescence assay based on determination of trypsin activity can detect rapidly *Vibrio parahaemolyticus* but cannot differentiate *Vibrio parahaemolyticus* from *Vibrio alginolyticus* or *Vibrio harvei*. The conventional methods for identification of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* are time-consuming because the 16S rRNA sequence reveals homology of 99.7% between *Vibrio parahaemolyticus* and *Vibrio alginolyticus*.

[0005] The genus *Vibrio* includes 37 different species, all of which are derived from aquatic environment. Based on the systematic data of rRNA, species known as *V. anguillarum*, *V. ordalii*, and *V. damsela* have been newly classified as *Listonella* or *Photobacterium*. Ten species are involved in gastroenteritis, infection of the wound, and human septicemia, while 7 species are known to be pathogens for fish. *Vibrio parahaemolyticus* occurs usually in an environment such as river-mouth and sea, being isolated from sea water and fishes and shellfishes often in summer.

[0006] For isolation and identification of *Vibrio parahaemolyticus*, the specimen is inoculated into a selective medium such as the bromothymolblue-teepol agar medium or TCBS agar medium, followed by isolation of bluish green colonies and examinations for the biochemical properties of the colonies. Unfortunately many *Vibrio* species show the same responses, and thus more detailed biochemical examinations are required for reliable identification. Examinations for a variety of biochemical properties on many isolates are time-consuming and laborious. Serological identification of *Vibrio parahaemolyticus* shows a cross reaction with other *Vibrio* species.

[0007] A method for identification of a *Vibrio* species was developed which used DNA. In this method were used DNA probes capable of amplifying the cholera toxin operon from *V. cholera* 01 to identify specifically the bacterium. These probes cross-react with *Vibrio* species other than cholera toxin-producing *V. cholera*. A method for identification of *V. vulnificus* has been developed in which hybridization is carried out on a membrane filter by using a fluorescent-labeled oligonucleotide probe (Wright, A.C. et al., Appl. Environ. Microbiol. 59: 541-546, 1993).

[0008] In addition, the oligonucleotide DNA probe was constructed from a portion of the cytolysin gene (*hlyA*) sequence of *V. vulnificus* and labeled through the covalent bond. These probes do not react with non-toxinogenic *V. vulnificus* and therefore do not detect all strains of *V. vulnificus*.

[0009] Similarly, other molecular biologic methods using the toxic factor (TDH, TRH) genes as the target can detect toxinogenic *V. parahaemolyticus*, though based on the toxic factor, all strains of *V. parahaemolyticus* cannot be detected.

## DISCLOSURE OF THE INVENTION

[0010] The object of this invention is to provide a method for differentiation of *Vibrio parahaemolyticus* from other 36 *Vibrio* species.

[0011] The object of this invention is to provide a method for detection of the 285-bp *gyrB* gene fragments specific for *Vibrio parahaemolyticus* by the PCR method without the necessity for DNA extraction or like operations from bacterial cells by use of *Vibrio parahaemolyticus*-specific primers.

[0012] Because this invention provides oligonucleotide probes useful for PCR, this invention relates to oligonucleotide primers for amplification of the target nucleotide sequence characteristic of *Vibrio parahaemolyticus*. The primers are exemplified by Sequences No.5 and No.6, which are used as a primer set for detection of the target nucleotide sequence characteristic of *Vibrio parahaemolyticus*.

[0013] The primer set is used in the method for determining whether or not there is the target nucleotide sequence characteristic of *Vibrio parahaemolyticus*. A *Vibrio parahaemolyticus*-specific primer is capable of detecting a *Vibrio*

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*parahaemolyticus*-specific *gyrB* gene fragment by the PCR method.

[0014] In this invention "primer" means an oligonucleotide which is produced synthetically or biologically and includes a specific nucleotide sequence which permits hybridization to a section containing the target nucleotide sequence.

[0015] A primer is capable of replicating a full target nucleotide sequence by synthesis by extension in the presence of polymerase or an analogous enzyme.

[0016] A primer is used in the method for amplification of nucleotide sequence, such as PCR and sequence displacement amplification (SDA). A particular primer, especially those useful for SDA technology, contains not only the sequence capable of hybridization to the target nucleic acid, but also a recognition sequence for restriction endonuclease and an arbitrary sequence which allows polymerase or another enzyme continuing polymerase-like activity to direct itself to initiate the synthesis of the template-specific oligonucleotide.

[0017] In this invention, "hybridization" means a process where, under pre-determined reaction conditions, partially or completely complementary nucleic acid chains, standing opposite to each other in an anti-parallel way, form a two-strand nucleic acid through specific and stable hydrogen bonds.

[0018] As described above, this invention relates to oligonucleotide primers useful for determination of the presence or absence of the target nucleotide sequence that is specific for *Vibrio parahaemolyticus*.

[0019] The procedures used for such determination include not only the PCR-based gene amplification procedure, but also Southern hybridization, a prior art, wherein the primer is used as a probe.

[0020] The primer in this invention is specific for the *gyrB* subunit gene sequence of *Vibrio parahaemolyticus*. The probe is specific for the internal consensus sequence in the primer amplification product.

[0021] The inventors have studied a method using the *gyrB* gene encoding the B subunit protein of DNA gyrase (topoisomerase II) as a highly specific probe, to solve the problems in the prior art described above.

[0022] A method has been reported recently for detection and taxonomic analysis of *Pseudomonas putida* with a universal primer with which the *gyrB* gene was sequenced directly. Such universal primers were used for PCR-based amplification of the *gyrB* gene fragments of various Gram-negative and Gram-positive bacteria. The inventors used these existing primers to amplify the 1.2-kb *gyrB* fragments of 37 *Vibrio* species. The *gyrB* base sequence of *Vibrio parahaemolyticus* ATCC17802 and that of *Vibrio alginolyticus* ATCC17749 (abbreviated as 'VA' somewhere hereinafter) have been shown.

[0023] In addition, the inventors prepared PCR primers capable of amplifying and identifying only the *gyrB* gene of *Vibrio parahaemolyticus*. The sensitivity of these *Vibrio parahaemolyticus*-specific primers was investigated with 118 *Vibrio parahaemolyticus* strains, 20 *Vibrio alginolyticus* strains, and other 78 strains.

[0024] Yamamoto and Harayama (Appl. Environ. Microbiol. 61: 1104-1109, 1995) and others prepared PCR primers capable of amplifying the *gyrB* gene from two conserved regions of the amino acid sequences of the DNA gyrase subunit B proteins. These primers were used for amplification of the about 1.2-kb *gyrB* gene fragments from various bacteria.

[0025] The *gyrB* gene fragment amplified from *Vibrio parahaemolyticus* ATCC17802 and that from *Vibrio alginolyticus* AATCC17749 were cloned by using a suitable vector according to the conventional method of recombination (Sambrook et al., Molecular cloning: a laboratory manual, 2<sup>nd</sup> ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 1989).

[0026] Desirable vectors include pGEMzf(+), and any common vector may be used.

[0027] The 1.2 kb *gyrB* gene fragment from *Vibrio parahaemolyticus* cloned with pGEMzf(+) is called a plasmid pVP *gyrB*, and that from *Vibrio alginolyticus* a plasmid pVAg *gyrB*.

[0028] The probe is amplified also with a conventional method (Sambrook et al., 1989). For example, a plasmid is inserted into the vector for transformation of *Escherichia coli* with an effective mean such as calcium chloride. Transformed cells are cultivated under appropriate conditions.

[0029] The target genes are collected after lysis of bacterial cells, and purified by the alkali method or the like. Purified plasmid is used as the specimen.

[0030] The PCR method was tried for detection and differentiation of *Vibrio parahaemolyticus* from other *Vibrio* species including *Vibrio alginolyticus*. This method is capable of amplifying a sequence homologous with the probe, practically increasing the sensitivity.

[0031] According to the base sequence of the probe, the synthesized oligonucleotide primer amplifies DNA only when the target base sequence is present in the specimen. Not only the sensitivity is enhanced but also an absolute specificity can be attained by using an oligonucleotide having specificity based on the base sequence of the DNA probe.

[0032] For preparation of effective primers, the base sequence of pVP *gyrB* and that of pVAg *gyrB* were determined with the DNA sequencer according to the conventional method.

[0033] For determination of the base sequence of *gyrB*, the base sequence of the N-terminal and C-terminal regions of the amplified fragment was also determined by using UP-1S and UP-2Sr primers (Yamamoto and Harayama, Appl. Environ. Microbiol. 61: 1104-1109, 1995). For extension of the determined base sequence, an additional primer was prepared from the base sequence determined by using UP-1S. The length of the whole base sequence of the amplified

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fragment is 1258 bp, and the sequence is shown in Sequence No.1 (pVP*gyrB*) and Sequence No.3 (pVAg*gyrB*). The amino acid sequence encoded by Sequence No.1 and that encoded by Sequence No.3 are shown in Sequence No.2 and Sequence No.4, respectively.

[0034] With this information of the base sequence, 21-bp primers were prepared that can detect and identify *Vibrio parahaemolyticus* from other bacteria. These primers contain Sequences No.5 and No.6, being usable as a primer set.

[0035] These novel primers are useful in the existing assay method using PCR (Saiki et al., Science 239: 487-491, 1988). These primers are used for amplification of the target DNA in a specimen, making the amount of DNA sufficient for detection. Following the amplification step, the step of detection may be performed by any method as far as it is effective for detection of DNA, for example, by electrophoresis on agarose gel.

[0036] The target DNA functions as the template. Amplification of the template DNA in the specimen is effected by treatment of the primer pair with a duplex DNA. This treatment results in extension of the sequence complementary to each nucleotide sequence. The resultant sequence functions as the template of the primer. The treatment process comprises denaturation of DNA, annealing to a sequence complementary to the primer, and extension of the primer with DNA polymerase (e.g. Taq polymerase), and is repeated until DNA has been produced in an amount sufficient for detection of the target sequence. The conditions of the amplification based on PCR are summarized in the Example 3 below.

[0037] Following amplification, the amplified sequence is detected by electrophoresis on agarose gel. The primer pair [VP1(Sequence No.5), VP2 (Sequence No.6)] amplifies the 285-bp when the *gyrB* gene sequence is used as the target.

[0038] The 285-bp DNA was amplified for 37 *Vibrio* strains obtained from ATCC, JCM, CDC, and NCIMB collections. The chromosomal DNA preparations from these bacteria were obtained according to the conventional method (Sambrook et al., Molecular cloning: a laboratory manual, 2<sup>nd</sup> ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 1989). The target DNA, 1 µg, was subjected to PCR. The 285-bp specific band was found only in the preparation from *Vibrio parahaemolyticus*, while it was not detected in any preparation from other species (Table 1). However the PCR-based amplification using the primer set (UP-1, UP-2r) according to Yamamoto and Harayama (1995) detected 1.2-kb *gyrB* fragment, and thus could confirm the presence of the DNA gyrase B subunit. Based on these findings, it is concluded that the primers of this invention are *Vibrio parahaemolyticus*-specific and usable for detection of the pathogen.

[0039] This invention relates to the oligonucleotides characterized in that they have the nucleotide sequence derived from Sequence No.1 and include at least one site capable of amplifying the *Vibrio parahaemolyticus*-specific nucleotide sequence. This invention relates to the oligonucleotides characterized in that they have the nucleotide sequence derived from Sequence No.1 but not from Sequence No.3 and include at least one site capable of amplifying the *Vibrio parahaemolyticus*-specific nucleotide sequence.

Table 1

S.no.	Microbes	Strain Number	PCR results of <i>gyrB</i>	
			1.2-kb	285bp
1	<i>Vibrio aestuarianus</i>	ATCC35048	+	-
2	<i>Vibrio albensis</i>	ATCC14547	+	-
3	<i>Vibrio alginolyticus</i>	ATCC17749	+	-
4	<i>Vibrio campbelli</i>	ATCC25920	+	-
5	<i>Vibrio carchariae</i>	ATCC35084	+	-
6	<i>Vibrio cholerae</i> 01	P1418	+	-
7	<i>Vibrio cholerae</i> non 01	NR	+	-
8	<i>Vibrio cincinnatiensis</i>	ATCC35912	+	-
9	<i>Vibrio costicola</i>	ATCC33508	+	-
10	<i>Vibrio diazotrophicus</i>	ATCC33466	+	-
11	<i>Vibrio fischeri</i>	ATCC 7744	+	-
12	<i>Vibrio fluvialis</i>	JCM3752	+	-
13	<i>Vibrio furnissii</i>	ATCC35016	+	-

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Table 1. (continued)

S.no.	Microbes	Strain Number	PCR results of gyrB	
			1.2-kb	285bp
14	<i>Vibrio gazogenes</i>	ATCC29988	+	-
15	<i>Vibrio harveyi</i>	ATCC14126	+	-
16	<i>Vibrio hollisae</i>	CDC75-80	+	-
17	<i>Vibrio logei</i>	ATCC29985	+	-
18	<i>Vibrio marinus</i>	ATCC15381	+	-
19	<i>Vibrio mediterranei</i>	ATCC43341	+	-
20	<i>Vibrio metschnikovii</i>	ATCC 7708	+	-
21	<i>Vibrio mimicus</i>	CNS9582	+	-
22	<i>Vibrio mytili</i>	NCIMB13275	+	-
23	<i>Vibrio natriegens</i>	ATCC14048	+	-
24	<i>Vibrio navarrensis</i>	NCIMB13120	+	-
25	<i>Vibrio nereis</i>	ATCC25917	+	-
26	<i>Vibrio nigripulchritudo</i>	ATCC27043	+	-
27	<i>Vibrio ordalii</i>	ATCC33509	+	-
28	<i>Vibrio orientalis</i>	ATCC33934	+	-
29	<i>Vibrio parahaemolyticus</i>	ATCC17802	+	+
30	<i>Vibrio proteolyticus</i>	ATCC15338	+	-
31	<i>Vibrio salmonicida</i>	ATCC43839	+	-
32	<i>Vibrio splendidus</i>	ATCC33125	+	-
33	<i>Vibrio tubiashii</i>	ATCC19109	+	-
34	<i>Vibrio vulnificus</i>	ATCC2046	+	-
35	<i>Listonella anguillarum</i>	ATCC19264	+	-
36	<i>Listonella pelagia</i>	ATCC25916	+	-
37	<i>Photobacterium damsela</i>	ATCC33539	+	-
ATCC : American Type Culture Collection JCM : Japan Collections of Microorganisms NCIMB : National Collections of Industrial and Marine Bacteria. CDC : Centre for Disease Control				

[0040] The symbols other than those described above refer to the strain names.

[0041] Also the standard strains of genera *Aeromonas*, *Alteromonas*, *Marinomonas*, *Shigella*, *Shewanella*, *Salmonella*, *Escherichia* and *Staphylococcus aureus* were examined for the presence of *gyrB* and the *Vibrio parahaemolyticus*-specific 285-bp. As shown in Table 2, *gyrB* was found to be present in all of the strains though the *Vibrio parahaemolyticus*-specific 285-bp amplification was found in none of the strains.

Table 2

S.no.	Microbes	Strain number	PCR results of gyrB	
			1.2-kb	285bp
1	<i>Alteromonas atlantica</i>	ATCC19262	+	-

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Table 2 (continued)

S.no.	Microbes	Strain number	PCR results of gyrB	
			1.2-kb	285bp
2	<i>Alteromonas carrogeenovara</i>	ATCC43555	+	-
3	<i>Alteromonas citrea</i>	ATCC29719	+	-
4	<i>Alteromonas espejiana</i>	ATCC29659	+	-
5	<i>Alteromonas haloplanktis</i>	ATCC14393	+	-
6	<i>Alteromonas luteoviolaceae</i>	ATCC33492	+	-
7	<i>Alteromonas macleodii</i>	ATCC27126	+	-
8	<i>Alteromonas tetraodonis</i>	NCIMB13177	+	-
9	<i>Alteromonas undina</i>	ATCC29660	+	-
10	<i>Marinomonas communis</i>	ATCC27118	+	-
11	<i>Marinomonas vaga</i>	ATCC27119	+	-
12	<i>Aeromonas hydrophila</i>	ATCC19570	+	-
13	<i>Escherichia coli</i>	ATCC25922	+	-
14	<i>Salmonella typhimurium</i>	ATCC13311	+	-
15	<i>Shewanella putrefaciens</i>	ATCC 8071	+	-
16	<i>Shigella dysenteriae</i>	ATCC13313	+	-
17	<i>Shigella sonnei</i>	ATCC29930	+	-
18	<i>Staphylococcus aureus</i>	ATCC12600	+	-

[0042] Various phenotypes, serotypes, and toxinogenic types have been reported for *Vibrio parahaemolyticus*. A probe specific for the thermostable hemolysin, a toxin produced by *Vibrio parahaemolyticus*, has been reported (Nishibuchi et al., FEMS Microbiol. Lett. 55: 251-256, 1990). Such toxin-specific probes are clinically important, though they cannot detect all types of *Vibrio parahaemolyticus*. For prevention of contamination of food with *Vibrio parahaemolyticus*, it is essential to detect all types of *Vibrio parahaemolyticus* in food or in the environment. Then *Vibrio parahaemolyticus* strains isolated from food, water, soil, and other materials were investigated for their phenotypes, serotypes, and the toxicity. All of the 118 *Vibrio parahaemolyticus* strains were subjected to PCR using the primers VP1 and VP2. It was evident that the 285-bp had been amplified.

[0043] The results are summarized in Table 3. The strains shown in Table 3 are those isolated from and identified in food, soil, water or feces, some of which were kindly given by Prof. Shinoda, Okayama University, and Prof. Yamamoto, Kyushu University. As shown in Table 3, amplification of the 285-bp was not observed in any of the 20 strains of *Vibrio alginolyticus* also isolated from various materials.

[0044] This invention relates to oligonucleotides characterized in that they have the nucleotide sequence obtained from Sequence No.1, contain at least one site capable of amplifying the nucleotide sequence characteristic of *Vibrio parahaemolyticus*, and cannot amplify the nucleotide sequence derived from *Vibrio alginolyticus* or *Vibrio harvei*.

[0045] This invention relates to oligonucleotides characterized in that they have the nucleotide sequence obtained from Sequence No.1 but not from Sequence No.3, contain at least one site capable of amplifying the nucleotide sequence characteristic of *Vibrio parahaemolyticus*, and cannot amplify the nucleotide sequence derived from *Vibrio alginolyticus* or *Vibrio harvei*.

Table 3

S.no.	Name	Strain no.	Kanagawa phenomenon	PCR
1	<i>V.parahaemolyticus</i>	33-7	+	+
2	<i>V.parahaemolyticus</i>	33-8	+	+
3	<i>V.parahaemolyticus</i>	33-10	+	+

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Table 3 (continued)

S.no	Name	Strain no.	Kanagawa phenomenon	PCR
4	<i>V. parahaemolyticus</i>	V83	+	+
5	<i>V. parahaemolyticus</i>	WP-1(y)	+	+
6	<i>V. parahaemolyticus</i>	WP-1	+	+
7	<i>V. parahaemolyticus</i>	39-11	-	+
8	<i>V. parahaemolyticus</i>	46-11	-	+
9	<i>V. parahaemolyticus</i>	AQ3301	-	+
10	<i>V. parahaemolyticus</i>	AQ3314	-	+
11	<i>V. parahaemolyticus</i>	AQ3321	-	+
12	<i>V. parahaemolyticus</i>	AQ3326	-	+
13	<i>V. parahaemolyticus</i>	AQ3331	-	+
14	<i>V. parahaemolyticus</i>	AQ3343	-	+
15	<i>V. parahaemolyticus</i>	AQ3345	-	+
16	<i>V. parahaemolyticus</i>	AQ3346	-	+
17	<i>V. parahaemolyticus</i>	AQ3354	-	+
18	<i>V. parahaemolyticus</i>	AQ3360	-	+
19	<i>V. parahaemolyticus</i>	AQ3627	-	+
20	<i>V. parahaemolyticus</i>	AQ3629	-	+
21	<i>V. parahaemolyticus</i>	AQ3633	-	+
22	<i>V. parahaemolyticus</i>	AQ3634	-	+
23	<i>V. parahaemolyticus</i>	BB22	-	+
24	<i>V. parahaemolyticus</i>	ML34	-	+
25	<i>V. parahaemolyticus</i>	ML159	-	+
26	<i>V. parahaemolyticus</i>	ML1017	-	+
27	<i>V. parahaemolyticus</i>	MY67-6	-	+
28	<i>V. parahaemolyticus</i>	MY73-2	-	+
29	<i>V. parahaemolyticus</i>	OK80-480	-	+
30	<i>V. parahaemolyticus</i>	OKA80-214	-	+
31	<i>V. parahaemolyticus</i>	OKA80-232	-	+
32	<i>V. parahaemolyticus</i>	S53	-	+
33	<i>V. parahaemolyticus</i>	RIMD2210521	-	+
34	<i>V. parahaemolyticus</i>	AR1-01	-	+
35	<i>V. parahaemolyticus</i>	AR3-02	-	+
36	<i>V. parahaemolyticus</i>	AR4-01	-	+
37	<i>V. parahaemolyticus</i>	AR4-02	-	+
38	<i>V. parahaemolyticus</i>	AR6-01	-	+
39	<i>V. parahaemolyticus</i>	AR6-02	-	+
40	<i>V. parahaemolyticus</i>	AR7-01	-	+
41	<i>V. parahaemolyticus</i>	KB1-01	-	+

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Table 3 (continued)

S.no.	Name	Strain no.	Kanagawa phenomenon	PCR
42	<i>V.parahaemolyticus</i>	KB1-03	-	+

[0046] For evaluation of the assay systems based on PCR, dilutions of the genomic DNA of *Vibrio parahaemolyticus* ATCC17802 strain were prepared and used as the template for PCR-based amplification. Even in the dilution containing only 1ng of the genomic DNA, detection was possible by amplification using the primers VP1 and VP2. For enhancement of the sensitivity from the ng level up to the pg level, DNA after electrophoresis was subjected to the Southern blotting. The dilution of cultivated *Vibrio parahaemolyticus* ATCC17802 cells was used for amplification with the primer described above, where the detection limit was about 1cfu/reaction tube. That is, the detection limit based on PCR was 1cfu/10  $\mu$ l or 10<sup>3</sup>cfu/ml. Detection by plating or by use of a selective agar medium has a sensitivity capable of detecting one liable cell but requires much labor and much time.

[0047] The assay methods based on PCR are superior to the conventional detection methods from the viewpoint of the balance among the speed, sensitivity, and specificity which is essential for the method of detection of bacteria, and thus useful.

BEST EMBODIMENTS OF THE INVENTION

[0048] This invention is explained with Examples in the following. The Examples show the mode of working of the invention, and do not limit the invention at all.

## Example 1

Conventional method of isolation of *Vibrio parahaemolyticus* in food

[0049] To 25 g of a food specimen, was added the alkaline peptone water [manufactured by Nissui Pharmaceutical Co., Ltd.] followed by inoculation with *Vibrio parahaemolyticus* ATCC17802 and incubation at 37°C for 18 hours. One loopful amount of the culture was inoculated into the TCBS agar medium by streaking and incubated at 37°C for 24 hours. All of green colonies were isolated further with the T<sub>1</sub>N<sub>1</sub> agar medium (distilled water containing 1% of Bacto tryptone, 1% of NaCl, and 1.5% of agar). Sufficiently isolated colonies were subjected to biochemical examinations. The strains that showed the following properties were identified as typical *Vibrio parahaemolyticus*: positive tests for oxidase, lysine decarboxylase, ornithine decarboxylase, gelatinase, lipase, and chitinase; indole producing; viable at the salt concentration of 0.5 to 8% at 42°C; sensitive to O/129 (150  $\mu$ g); producing acids from glucose, mannitol, and mannose; negative tests for arginine dehydrogenase and arginase; not viable at salt concentration of 0%; and producing no acid from sucrose, lactose or salicin.

## Example 2

Isolation of chromosomal DNA

[0050] A *Vibrio parahaemolyticus* strain was cultured by shaking in the TSB medium (manufactured by Eiken Chemical Co., Ltd.) at 37°C for about 24 hours. Cells were collected by centrifugation (manufactured by Tomy Seiko Co., Ltd.) (15,000 rpm, 15 minutes, 4°C), and suspended in 10 ml of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Cells were lysed by lysozyme (final concentration 1 mg/ml; manufactured by Wako Pure Chemical Industries, Ltd.), and maintained at 37°C for 20 minutes while being shaken gently. To the cell lysate was added SDS (final concentration 0.5%) and the mixture was incubated at 65 °C. For degradation of protein and RNA, were added proteinase K (final concentration 500  $\mu$ g/ml) and RNase (final concentration 5  $\mu$ g/ml), and the mixture was incubated at 37°C for 30 minutes and for 60 minutes, respectively. The samples were extracted three times with buffer saturated phenol (GIBCO/BRL), once with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1). Clear supernatant was obtained by centrifugation of the extract and DNA was precipitated by addition of two volumes of ice-cooled ethanol: 3M sodium acetate (10:1). The reaction mixture was kept overnight at -20°C. The DNA precipitate was concentrated by centrifugation, and ethanol was evaporated off under reduced pressure. The dried DNA was dissolved in the TE buffer, which was used as the DNA template. The purity of DNA was determined by electrophoresis on agarose gel, and the concentration of DNA was determined with the spectrophotometer.



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## Example 3

Assay based on PCR

## 5 Preparation of specimens

[0051] All cells including also those not used for extraction of DNA were used as the template. Fresh cells grown on agar media were used. Cells grown on liquid media were used after separation of cells by centrifugation followed by washing once with PBS buffer (pH 7.5), and a suitable number of resultant cells were used. In some cases, DNA  
10 extracted with phenol-chloroform was used as the template of PCR amplification. PCR amplification conditions

[0052] PCR assay was carried out with a DNA Thermal Cycler (Perkin Elmer Corp.). One hundred microliter of the reaction mixture (Tris-HCL 100 mM, MgCl<sub>2</sub> 15 mM, KCl 500 mM, pH 8.3) contains 100 ng of genomic DNA, 200  $\mu$  M of dNTPs, and 1  $\mu$  M of primer. DNA degeneration, annealing, and DNA extension were carried out at 94°C for 60 seconds, at 60°C for 60 seconds, and at 72°C for 120 seconds, respectively, and a total of 30 cycles of amplification were  
15 performed. Following amplification, detection was made by gel electrophoresis. Twenty microliter of the sample was subjected to electrophoresis on agarose gel (1% agarose, SeaKem ME, FMC Bioproducts, Rockland, Maine). The DNA band was stained with the ethidium bromide solution for 10 minutes and observed under ultraviolet irradiation.

INDUSTRIAL APPLICABILITY

20 [0053] A primer which reacts specifically with a *gyrB* gene of *Vibrio parahaemolyticus* to thereby differentiate and identify the same among other *Vibrios* and strains other than the genus *Vibrio* could be provided.

[0054] The *Vibrio parahaemolyticus*-specific primer serves to detect 285-bp *gyrB* gene fragments specific for this *Vibrio* by the PCR method without the necessity for DNA extraction or like operations from bacterial cells.

## List of Sequences

## Sequence No.: 1

30 Sequence length: 1258

Sequence type: nucleic acid

35 Strand number: single strand

Topology: straight chain

40 Sequence class: genomic DNA

## Sequence

45 GAAGTCATCA TGACCGTTCT GCATGCOGGT GGTAAATTOG ATGATAACTC GTACAAAGTA 60

TCAGGOGGTC TTCACGGOGT GGGTGTITTOG GTAGTAAACG CACTGTCAGA AAAAGTGGTA 120

CTAACCATCC ATOGTGGOGG TCATATCCAC ACGCAAACCT ACOGTCATGG TGAGCCTGAA 180

50 AOGCCTCTAG OGGTGTGTTGG TGATGOGGAT AAAACTGGTA CACAAATTOG TTTCTGGOCA 240

AGTGCAGAAA CTTTCTCTAA CACTGAATTC CATTAGACA TOCTAGCAAA ACGTCTGOGT 300

55 GAGCTATOGT TCTTGAACCT AGGOGTTTCT ATCAAGCTTA TTGATGAGOG OGAAGOGGAC 360

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5 AAGCAAGATC ACTTCATGTA TGAAGGTGGT ATTCAAGOGT TGGTTCAGCA CTAAACACC 420  
AACAAAACAC CAATCATOGA GAAAATCTTC CACTTOGACT TAGAAOGTGA AGAOGGCATT 480  
TOGGTAGAAG TGGCAATGCA GTGGAAOGAT GGTTTCCAAG AGAACATCTT CTGTTTCACC 540  
10 AACACATTC CACAGOGOGA TGGTGGTACT CAOCTTGCTG GTTTOOGTGC GGCAATAACA 600  
OGTAOGCTAA ACAGCTTTAT GGATAAGAA GGCTTCTOGA AGAAAGOGAA AAOGGCAAOG 660  
TCAGGOGAOG ATGOGOGTGA AGGTTTGA CTGTTTGTGTT CAGTAAAAGT GOCTGATOCA 720  
15 AAATCTCTOGA GOCAAAACAA AGACAAACTG GTTCTCTCTG AAGTGAAATC AGOGGTTGAA 780  
TOGGOGATGG GTGAGAAGCT ATCTGAGTTC TTGGTGGAAA AOOCAAGTGA AGOGAAAATG 840  
20 GTTGTGTGTA AAATCATOGA TGCAGCAOGT GCAOGTGAAG COGCAOGTAA AGOGOGTGAA 900  
ATGACTOGTC GTAAAGGOGC GCTAGACCTA GCAGGOGTAC CAGGCAAACT TGCAGACTGT 960  
CAGGAAAAAG ATOOGGCACT CTCTGAACTA TACATTGTGG AGGGTGACTC TGOGGGTGGT 1020  
25 TCAGCTAAGC AGGGTGGTAA TOGTAAGAAT CAGGCAATOC TAOCACTGAA AGGTAAGATC 1080  
CTGAAOGTAG AAAAAGCAOG TTTOGACAAG ATGTTGTCTT OGCAAGAAGT TGCAAOGCTT 1140  
30 ATTACAGCAC TTGGCTGTGG TATOGGTGTG GAOGAGCACA AOOOGGACAA ACTGOGTTAC 1200  
CACACATCA TCATCATGAC OGAOGCAGAC GTAGAGGCTC GCACATOOGT AOOCTGCT 1258

35 Sequence No.: 2

Sequence length: 419

40 Sequence type: amino acid

Topology: straight chain

45 Sequence class:

Sequence

50 EVIMTVLHAG GKFDNSYKV SGGLHGVGS VVNALSEKV LTHRGGHH TQTYRHGEPE 60  
TFLAVVGDAK KTGTRQRFWP SAETFSNTEF HYDLAKRLR ELSFLNSGVS IKLDEREAD 120  
KQDHFMYEGG IQAFVQHLNT NKTPLEKIF HFDLEREDGI SVEVAMQWND GFQENIFCFT 180

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NNIPQRDGGT HLAGFRAAIT RILNSFMDKE GFSKKARTAT SGDDAREGLT AVVSVKVPDP 240  
KFSSQTKDKL VSSEKSAVE SAMGEKLSEF LVENPSEAKM VCSKIIDAAR AREAARKARE 300  
MTRRKGALDL AGLPGKLADC QEKDPALSEL YIVEGDSAGG SARQGRNRKN QAILPLKIKI 360  
LNVEKARFDK MLSSQEVATL ITALGOGGR DEHNPDKLRY HNIIMTDAD VEARTSVPC 419

Sequence No.: 3

Sequence length: 1258

Sequence type: nucleic acid

Strand number: single strand

Topology: straight chain

Sequence class: genomic DNA

Sequence

GAAGTCATCA TGACGGTCTT GCATGCAGGT GGTAATTOG ACGATAACAC AAACAAATTA 60  
TOGGGTGGTC TOCAGGGGT ACGTGTCTCA GTAATAAAG CACTATCAGA GAAAGTTGAG 120  
CTAAGATTG ATGTGGTGG CATATOCAT ACGCAAACT ACGGOCATGG TGAGCTGCA 180  
ACGOCCTAG CCGTGTGGG TGATAAGGAT AAAACGGTA CACAAATTOG TTTCTGGCCA 240  
AGTGGGAGA CGTCTCTAA CACTGAGTC CACTATGACA TTCTGGGAA ACGCTGGGT 300  
GAACTGTCAT TOCTGAATC TGGTGTGTG ATCAAATGG TTGATGAAG TGAAGCGGAC 360  
AAACATGATC ACTTCATGTA TGAAGGTGGT ATTCAAGGT TCGTTGATCA OCTAAACACC 420  
AACAAAAGC CAATCATOGA GAGGGTCTC CACTTAACT CTGAGGTGA AGAAGGCAAT 480  
TCAGTTGAAG TGGGATGCA ATGGAAGAT GGTTCOAAG AGAACATCTT CTGCTTTACC 540  
AACAAATOC CACAGGTGA TGGTGGTACT CACCTGCTG GTTTCGTGC TGOGCTAACA 600  
CGTACATTGA ACAGCTTTAT GGATAAAGAA GGTTCCTGA AGAAAGGAA AACAGGACT 660  
TCAGGGAAG ATGCGGTGA AGGTCTAACT GCGGTGTTT OGGTGAAAGT GCGTATGCT 720  
AAGTCTCAA GCAAAACAAA AGACAAACTG GTTCTCTG AAGTGAAATC AGCTGTTGAG 780

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TCTGCAATGG GTGAAAACT GTCTGAGTTC TTGATTGAGA ACOOGACAGA AGOGAAGATG 840  
 GTTTGTTOGA AAATCATCAA TGCAGCACGT GCATCTGAAG CAGOGCTAA AGCTOGTGAA 900  
 ATGAOGGOC GTAAAGGTGC ACTAGACCTA GCAGGCTTC CAGGTAAAGT TGCAGACTGT 960  
 CAGGAAAAAG ATOGGCACT CTTGAACTA TACATAGTGG AGGGTGAATC GGCAGGCGGT 1020  
 TOOGCAAAC AAGGCGTAA CGTAAGAAC CAAGGATCA CACGCTAAA AGGTAAGATT 1080  
 CITAACTAG AAAAAGCAAG TTTOGACAAG ATGCTATCTT CTCTAGAAGT AGTAACACTG 1140  
 ATCAOOGCAT TAGGTTGTGG TATOGGTGTT GAGGAGGACA ACOOGGACAA GOCTOGGGAC 1200  
 CACAACATAA TCATCATGAC CGAOGCAGAC GTAGAGGCTC GCACATTOGT ACOCTGCT 1258

Sequence No.: 4

Sequence length: 419

Sequence type: amino acid

Topology: straight chain

Sequence class:

Sequence

EVIMTVLHAG GKFDNTNKL SGGLHGVRVS VINALSEKVE LTIHRGGHIH TQTYRHGEPA 60  
 TPLAVVGDTD KTGITQIRFWP SAETFSNTEF HYDILAKRLR ELSFLNSGVS IKLVDEREAD 120  
 KHDHFMYEGG IQAFVDHLNT NKIPIERVF HFNSEREDGI SVEVAMQWND GFQENIFCFT 180  
 NNIPQRDGGT HLAGFRAALT RILNSFMDKE GFSKKAKTAT SGDDAREGLT AVSVKVPDP 240  
 KFSSQTKDKL VSEVKSAVE SAMGEKLSEF LIENPTEAKM VCSKIINAAR ASEAPKARE 300  
 MTRRKALDL AGLPGKVADC QEKDPALFEL YVEGESAGG SAKQGRNRKN QAITPLKGKI 360  
 LNVEKARFDK MLSSLEVVTI TALGOGIGR DEDNPKPRD HNIIMTDAD VEARTSVPC 419

Sequence No.: 5

Sequence length: 21

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Sequence type: nucleic acid

Strand number: single strand

Topology: straight chain

Sequence class: genomic DNA

Sequence

CGG CGT GGG TGT TTC GGT AGT

Sequence No.: 6

Sequence length: 21

Sequence type: nucleic acid

Strand number: single strand

Topology: straight chain

Sequence class: genomic DNA

Sequence

TCC GCT TCG CGC TCA TCA ATA

## 40 Claims

1. An oligonucleotide having a nucleotide sequence derived from Sequence No.1, characterized in that it contains at least one site capable of amplifying a nucleotide sequence characteristic of *Vibrio parahaemolyticus*.
2. The oligonucleotide of Claim 1, having a nucleotide sequence unavailable from Sequence No.3.
3. The oligonucleotide of Claim 1 or Claim 2, incapable of amplifying nucleotide sequences originating in *Vibrio alginolyticus* and *Vibrio harvei*.
4. The oligonucleotide of Claim 1 or Claim 2 or Claim 3, represented by Sequence No.5 or Sequence No.6.
5. A method of detecting *Vibrio parahaemolyticus* by preparing a primer set comprising two of those of Claims 1 to 4, selectively amplifying therewith a DNA gyrase subunit B gene sequence contained in a specimen as a target, and determining whether or not there is a *gyrB* unit specific for *Vibrio parahaemolyticus* in the specimen.

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/00991

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl<sup>6</sup> C12N15/11, C12Q1/68, C12Q1/04, C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl<sup>6</sup> C12N15/11, C12Q1/68, C12Q1/04, C07H21/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSYS, MEDLINE, GENETYX

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 7-213299, A (Marine Biotechnology Institute Co., Ltd.), August 15, 1995 (15. 08. 95) (Family: none)	1 - 5
Y	Applied and Environmental Microbiology, Vol. 61(3) (1995) S. Yamamoto et al. "PCR Amplification and Direct Sequencing of gyrB Genes with Universal Primers and Their Application to the Detection and Taxonomic Analysis of Pseudomonas putida Strains" p. 1104-1109	1 - 5
Y	JP, 4-262799, A (Toyobo Co., Ltd.), September 18, 1992 (18. 09. 92) (Family: none)	1 - 5
Y	JP, 7-114719, B (Shimadzu Corp.), December 13, 1995 (13. 12. 95) (Family: none)	1 - 5
Y	JP, 5-123197, A (Shimadzu Corp.), May 21, 1993 (21. 05. 93) (Family: none)	1 - 5

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

\* Special categories of cited documents:

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Date of the actual completion of the international search

May 26, 1997 (26. 05. 97)

Date of mailing of the international search report

June 3, 1997 (03. 06. 97)

Name and mailing address of the ISA/

Japanese Patent Office

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/00991

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Microbial Pathogenesis, Vol. 18 (1995) J. Okuda et al. "Distribution of the cytolethal distending toxin A gene(cdtA) among species of Shigella and Vibrio, and cloning and sequencing of the cdt gene from Shigella dysenteriae" p. 167-172	1 - 5

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